Patent Application
Docket No. SPO-120

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Eric S. Olson
Art Unit	:	1623
Applicant	:	Tadao Saito, Haruki Kitazawa
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For	:	Phosphorylated Dextrans

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

EXPERT DECLARATION OF DR. HARUKI KITAZAWA UNDER 37 CFR §1.132

Sir:

I, Haruki Kitazawa, hereby declare:

THAT, I am a co-inventor on the subject application;

THAT, I am an Associate Professor at Tohoku University and, as can be seen from the attached professional history, I have extensive experience in the fields of biochemistry and immunology;

THAT, I have reviewed the Office Action mailed May 16, 2008, along with the references cited therein;

And being thus duly qualified, do further declare as follows:

1. The attached experimental results show the effects of the claimed phosphorylated dextrans in preventive studies. Specifically, the mouse studies demonstrate that the phosphorylated dextrans of the present invention have the ability of reducing allergic reactions *in vivo*. As shown in Figure 2, the cells in the OVA group, which had been sensitized with OVA, exhibited very strong proliferation activity against the OVA antigen stimulation, while the activity was decreased in the cells of the P-Dex group, which had been administered phosphorylated dextran together with OVA.

2. The preventive effects of phosphorylated dextrans are also evidenced by an attenuation of CD86 expression on B-cells and dendritic cells as shown in Figure 3 and presented numerically in Table 1. When the cells of G4 group ($CD11c^-CD8alpha^-$ cells; B cells, macrophages, *etc.*) were stimulated with OVA *in vitro*, the CD86 expression was increased in both of the OVA and P-Dex groups as compared to the control group; however, the CD86 expression tended to be lower in the P-Dex group compared to the OVA group. Similarly, in the G5 group ($CD11c^+CD8alpha^-$ cells; $CD8alpha^-$ dendritic cells), OVA stimulation lead to the elevated expression of CD86 in the two groups, but the CD86 expression was decreased in the P-Dex group compared to the OVA group in the Day 25 group.

3. The preventive effects of phosphorylated dextrans are also corroborated by the semi-quantitative PCR analysis of cytokine mRNAs as shown in Figures 4 to 6. The expression analysis of Th1 cytokines is illustrated in Figure 4. Therein, administration of phosphorylated dextrans alongside OVA produced significant increases over the control group and the OVA group in mRNA levels of IFN-gamma in the Day 20 group. IL-12p40 mRNA levels were also significantly higher in the group treated with phosphorylated dextrans compared to the OVA group in the Day 20 group. Figure 5 details the preventive effects of phosphorylated dextrans on the Th2 cytokines. The mRNA levels of IL-4, IL-5, and IL-6 were increased by OVA stimulation in both the OVA and P-Dex groups. The mRNA levels of IL-10 in the group treated with phosphorylated dextrans were higher than those of the control and OVA groups. Figure 6 shows the preventive effects on other

cytokines. The expression of IL-2 mRNA was more induced in the P-Dex group compared to the control group.

4. Finally, the preventive effects of phosphorylated dextrans are further substantiated by real-time quantitative PCR analysis of the cytokine expression. This example demonstrates the strongly induced levels of IFN-gamma mRNA in the group treated with phosphorylated dextrans when compared to the control group or the group sensitized with OVA antigen. Similar effects are shown when assaying for the levels of IL-4 and IL-10 mRNA.

5. These results show that phosphorylated dextran administration has preventive effects on diseases that may occur due to the imbalance of Th1 and Th2 cytokines, such as allergic conditions via the activation of Th1 cells as well as regulatory cells and IL-10.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

By: Haruki Kitazawa
Haruki Kitazawa

Date: Oct. 15, 2008

Attachments: Professional History of Haruki Kitazawa
Experimental Results

Haruki KITAZAWA - History -

EDUCATION

1993	**Ph. D.** Laboratory of Animal Products Chemistry, Faculty of Agriculture, Tohoku University, Sendai, 981 Japan
March 1988	**M. Sc.** Laboratory of Animal Products Chemistry, Faculty of Agriculture, Tohoku University, Sendai, 981 Japan
March 1986	**B. Sc.** Laboratory of Animal Products Chemistry, Faculty of Agriculture, Tohoku University, Sendai, 981 Japan

APPOINTMENTS

April 2002 - present **Associate Professor**

Laboratory of Animal Products Chemistry, Graduate School of Agriculture, Tohoku University, Sendai, 981 Japan

May 1996 - May 2002 **Senior Assistant Professor**

Laboratory of Animal Products Chemistry, Graduate School of Agriculture, Tohoku University, Sendai, 981 Japan

October 1994 - April 1996 **Postdoctoral Fellow**

JSPS Postdoctoral Fellowships for Research Abroad Program
Laboratory of Molecular Immunoreguration
NCI-FCRDC
Frederick, MD 21702

April 1990 - September 1994 **Assistant Professor**

Laboratory of Animal Products Chemistry, Faculty of Agriculture,
Tohoku University, Sendai, 981 Japan

April 1988 - March 1990 **Research Associate**

Laboratory of Animal Products Chemistry, Faculty of Agriculture,
Tohoku University, Sendai, 981 Japan

April 1986 - March 1988 **Master Course**

Laboratory of Animal Products Chemistry, Faculty of Agriculture,
Tohoku University, Sendai, 981 Japan

GRANT OBTAINED

2001-03	Grant-in-Aid for Scientific Research (B) (Number13556018) from the Ministry of Education, Science and Culture of Japan.
2001-02	Grant-in-Aid for Scientific Research (C) (Number13660265) from the Ministry of Education, Science and Culture of Japan.
2000	Grant from Uehara Memorial Foundation.
1999-00	Grant-in-Aid for Scientific Research (C) (Number11660265) from the Ministry of Education, Science and Culture of Japan.
1997-98	Grant-in-Aid for Scientific Research (C) (Number09660285) from the Ministry of Education, Science and Culture of Japan.
1996	Grant-in-Aid for encouragement of young scientists (Number09660285) from the Ministry of Education, Science and Culture of Japan.
1994-1996	JSPS Postdoctoral Fellowship Fund for Research abroad Program.
1994	Grant-in-Aid for encouragement of young scientists

(Number06760231) from the Ministry of Education, Science and Culture of Japan.

1993 Grant-in-Aid for encouragement of young scientists (Number05760201) from the Ministry of Education, Science and Culture of Japan.
Grant from Morinaga Hoshikai

1992 Grant-in-Aid for encouragement of young scientists (Number04760193) from the Ministry of Education, Science and Culture of Japan.
Grant from Morinaga Hoshikai

1991 Grant from Morinaga Hoshikai

1990 Grant-in-Aid for encouragement of young scientists (Number02760162) from the Ministry of Education, Science and Culture of Japan.

1989 Grant-in-Aid for encouragement of young scientists (Number01760224) from the Ministry of Education, Science and Culture of Japan.

AWARD

March 1994 Award for Young Scientist from The Japanese Society of Zootechnical Science.

PROFESSIONAL SOCIETIES

The Japanese Society of Zootechnical Science

The Japanese Society of Bioscience Biotechnology and Agrochemistry

The Japanese Society of Immunology

The American Dairy Science Association

TOP

Experimental Results

(1) Immunization Schedule with OVA

Mice were sensitized according to the immunization schedule shown in Fig. 1. Specifically, 500 µl of PBS containing 10 µg of OVA and 3 mg of $Al(OH_3)$ was intraperitoneally injected to each mouse on days 1, 8, and 15. The same volume of PBS was injected to a control group. At the same time, 2 mg of phosphorylated dextran suspended in 500 µl of distilled water was directly administered to the stomach of each mouse using a gastric tube over a period of consecutive 15 days from day 1 to day 15. The same volume of distilled water was injected to a control group. The mice were anesthetized with ether and then sacrificed by exsanguination on day 20 or 25, respectively, which corresponds to fifth or tenth day from the last administration of phosphorylated dextran. The body weight of the mice was measured and their serum was collected on days 0, 16, 20, and 25.

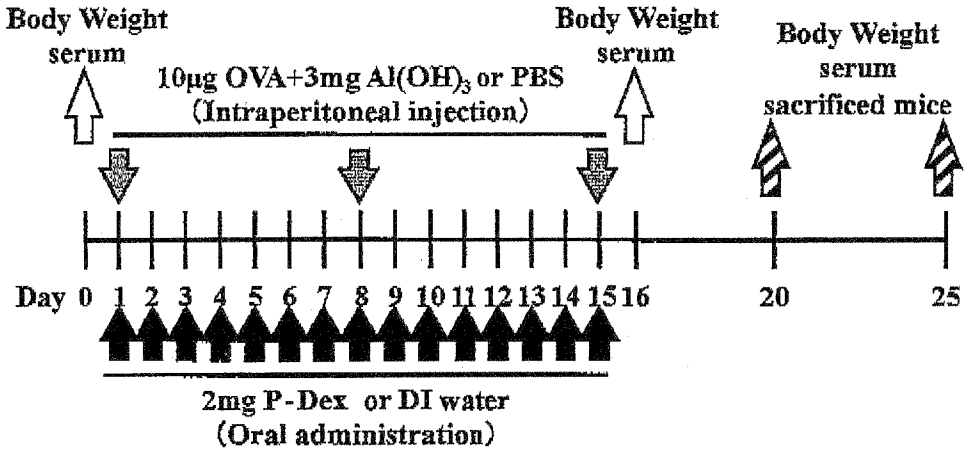

Fig. 1 Immunization schedule with OVA

(2) Cell proliferation against OVA antigen in preventive study

Spleen cells were prepared from the mice in each sensitized group. After 48 hours of *in vitro* stimulation with the OVA antigen (100 µg/ml) or without stimulation, the cells were assessed for proliferation activity. The obtained results are represented as cpm (counts per minute) for each animal in plot graphs.

When no stimulation was added, the cell proliferation activity was higher in both of the OVA and P-Dex groups which were sensitized with OVA, as compared to the control group. When stimulated with the OVA antigen, the cells in the OVA group exhibited very strong

proliferation activity, while the activity was decreased in the P-Dex group. This tendency was essentially the same between the groups sacrificed on days 20 and 25 (Fig. 2).

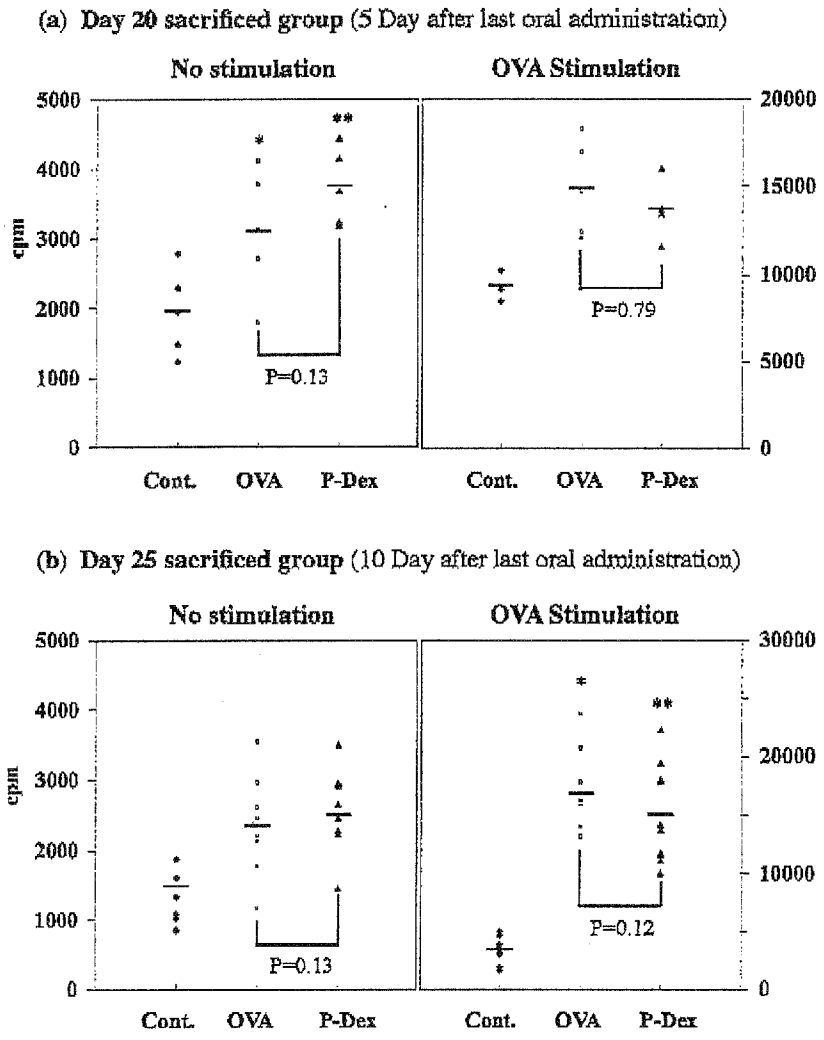

Fig. 2 Cell proliferation against OVA antigen in preventive study

*P<0.05, **P<0.01, and ***P<0.001 against control group.

(3) Expression of CD86 in preventive study

Spleen cells were prepared from the mice in each sensitized group. After 18 hours of *in vitro* stimulation with the OVA antigen (100 μg/ml) or without stimulation, the cells were analyzed for CD86 expression by flow cytometry. Specifically, the cells were fractionated into

four populations using two types of markers, CD11c and CD8α, and the expression of CD86 in each cell population was represented as a histogram. The cells were assessed based on the proportion of the cell population expressing CD86 at a prominently high level ($CD86^+$ cells) to the whole cells (Fig. 3) and the mean fluorescence intensity (Table 1). The cells in G7 group ($CD11c^-CD8\alpha^+$ cells) were not analyzed because they correspond to $CD8\alpha^+$ T cells and are considered not to express CD86.

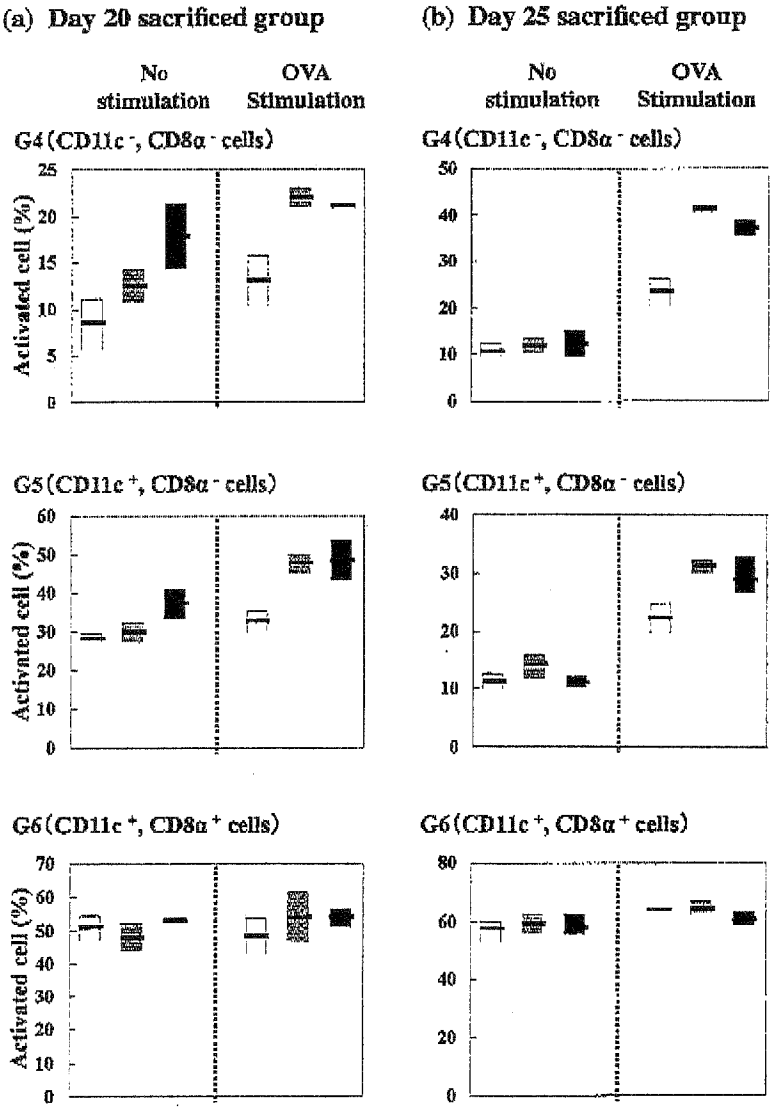

Fig. 3 Expression of CD86 in preventive study

Control group OVA group P-Dex group

Table 1 Expression of CD86 in preventive study

(a) G4 (CD11c$^-$, CD8α$^-$ cells)

Group	Stimuli	Day 20		Day 25	
		CD86$^+$ cell (%) mean (range)	MFI* mean (range)	CD86$^+$ cell (%) mean (range)	MFI mean (range)
Cont.		8.4 (5.7-11.2)	153.0 (146-160)	10.7 (9.6-12.4)	262.6 (261-265)
OVA		12.6 (10.8-14.3)	161.9 (157-167)	11.8 (10.1-13.5)	267.9 (251-281)
P-Dex		17.8 (14.4-21.3)	176.0 (167-186)	12.2 (9.7-14.8)	333.0 (261-292)
Cont.	OVA	13.0 (10.2-15.8)	164.8 (159-170)	23.5 (20.5-26.3)	366.3 (361-370)
OVA	OVA	21.9 (20.8-23.0)	203.6 (202-206)	41.0 (40.36-41.7)	580.4 (562-571)
P-Dex	OVA	20.9 (20.8-21.1)	197.5 (195-200)	37.1 (35.3-38.9)	611.4 (568-640)

(b) G5 (CD11c$^+$, CD8α$^-$ cells)

Group	Stimuli	Day 20		Day 25	
		CD86$^+$ cell (%)	MFI	CD86$^+$ cell (%)	MFI
Cont.		28.3 (27.3-29.3)	139.4 (132-147)	11.3 (10.0-12.7)	404.2 (377-428)
OVA		29.6 (27.1-32.0)	138.5 (130-147)	14.1 (11.7-15.9)	461.0 (387-523)
P-Dex		37.1 (33.5-40.8)	212.6 (146-279)	11.1 (10.0-12.2)	493.7 (440-536)
Cont.	OVA	32.3 (29.7-35.0)	197.3 (186-208)	22.0 (19.5-24.8)	521.9 (498-536)
OVA	OVA	47.6 (45.0-50.1)	281.3 (274-289)	30.9 (30.0-32.1)	554.3 (521-580)
P-Dex	OVA	48.3 (43.4-53.3)	294.8 (285-305)	28.8 (26.3-32.5)	674.7 (654-693)

(c) G6 (CD11c$^+$, CD8α$^+$ cells)

Group	Stimuli	Day 20		Day 25	
		CD86$^+$ cell (%)	MFI	CD86$^+$ cell (%)	MFI
Cont.		50.8 (47.1-54.5)	263.8 (262-266)	57.3 (52.9-60.0)	204.5 (189-221)
OVA		47.9 (43.9-51.8)	282.6 (262-303)	58.9 (55.9-62.4)	239.2 (219-251)
P-Dex		52.6 (51.9-53.2)	404.5 (304-505)	57.6 (55.3-62.1)	301.5 (233-412)
Cont.	OVA	48.2 (42.8-53.6)	515.1 (490-540)	63.8 (63.5-64.1	533.0 (438-654)
OVA	OVA	53.8 (46.5-61.0)	801.7 (728-875)	64.2 (62.7-67.1)	617.7 (554-716)
P-Dex	OVA	53.6 (50.8-56.4)	778.7 (736-822)	60.2 (58.7-63.2)	741.9 (560-907)

*MFI : mean fluorescence intensity

When the cells of G4 group (CD11c$^-$CD8α$^-$ cells; B cells, macrophages, *etc.*) were stimulated with OVA *in vitro*, the CD86 expression was increased in both of the OVA and P-Dex groups which were sensitized with OVA, as compared to the control group, regardless of the day of sacrifice (day 20 or 25). The CD86 expression tended to be lower in the P-Dex

group compared to the OVA group. In the G5 group ($CD11c^+CD8\alpha^-$ cells; $CD8\alpha^-$ dendritic cells), OVA stimulation lead to the elevated expression of CD86 in the two groups sensitized with OVA as compared to the control group, and the CD86 expression was decreased in the P-Dex group compared to the OVA group in the group sacrificed on day 25. On the other hand, the cells in G6 group ($CD11c^+CD8\alpha^+$ cells; $CD8\alpha^+$ dendritic cells) showed no such significant alterations as compared to the G4 and G5 groups on both days 20 and 25.

(4) Gene expressions of cytokines in preventive study

Spleen cells were prepared from the mice in each sensitized group. After 6 hours of *in vitro* stimulation with OVA antigen (100 μg/ml) or without stimulation, the cells were analyzed for cytokine mRNAs by semi-quantitative PCR. Specifically, the agarose gel electrophoresis pattern for each cytokine was analyzed using Scion Image and Relative Index was calculated by taking as 1 the intensity of the control group (with or without OVA stimulation) sacrificed on day 20. The results of expression analysis for the Th1 cytokines, IFN-γ, IL-12p40, IL-18, and TNF-α are shown in Fig. 4; the results for the Th2 cytokines, IL-4, IL-5, and IL-6, and the suppressive cytokine IL-10 are shown in Fig. 5; and the results for other cytokines, IFN-α, IL-1α, IL-1β, and IL-2 are shown in Fig. 6.

In vitro OVA stimulation tended to induce the expression of IFN-γ mRNA in all groups. In the group sacrificed on day 20, the IFN-γ mRNA expression was induced in the P-Dex group as compared to the control and OVA groups. The expression of IL-12p40 mRNA was induced in the P-Dex group as compared to the OVA group in the group sacrificed on day 20. The expression of TNF-α mRNA was induced by *in vitro* OVA stimulation in all groups, but there was no significant difference between each group.

In both groups sacrificed on days 20 and 25, *in vitro* OVA stimulation strongly induced the mRNA expression of IL-4, IL-5, and IL-6 in the two groups which were sensitized with OVA. The expression of IL-4 mRNA was induced in the P-Dex group as compared to the control and OVA groups, while the expression level of IL-6 in the P-Dex group was comparable to those in the other two groups. The IL-10 mRNA expression was induced by *in vitro* OVA stimulation in the P-Dex group as compared to the control and OVA groups, regardless of the day of sacrifice (day 20 or 25).

The mRNA expression levels of IL-1α and IL-1β in the P-Dex group were comparable to those in the control and OVA groups. *In vitro* OVA stimulation increased the mRNA expression levels in all groups. The IL-2 mRNA expression was not detectable when no stimulation was added, while the expression was induced by *in vitro* OVA stimulation in all groups. The expression of IL-2 mRNA was more induced in the P-Dex group compared to the

control group. The expression level of IFN-α mRNA in the P-Dex group was comparable to that in the control group.

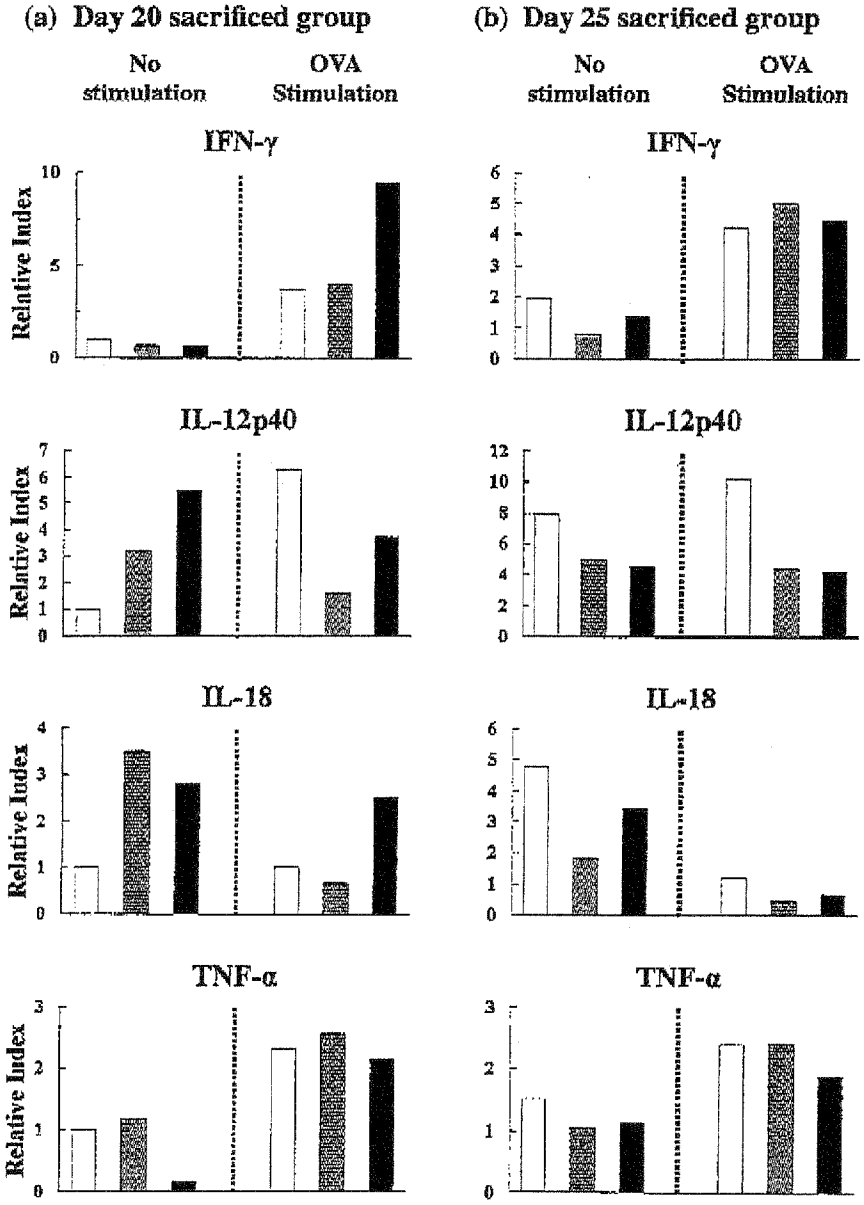

Fig. 4 Gene expressions of Th1 type cytokines in preventive study

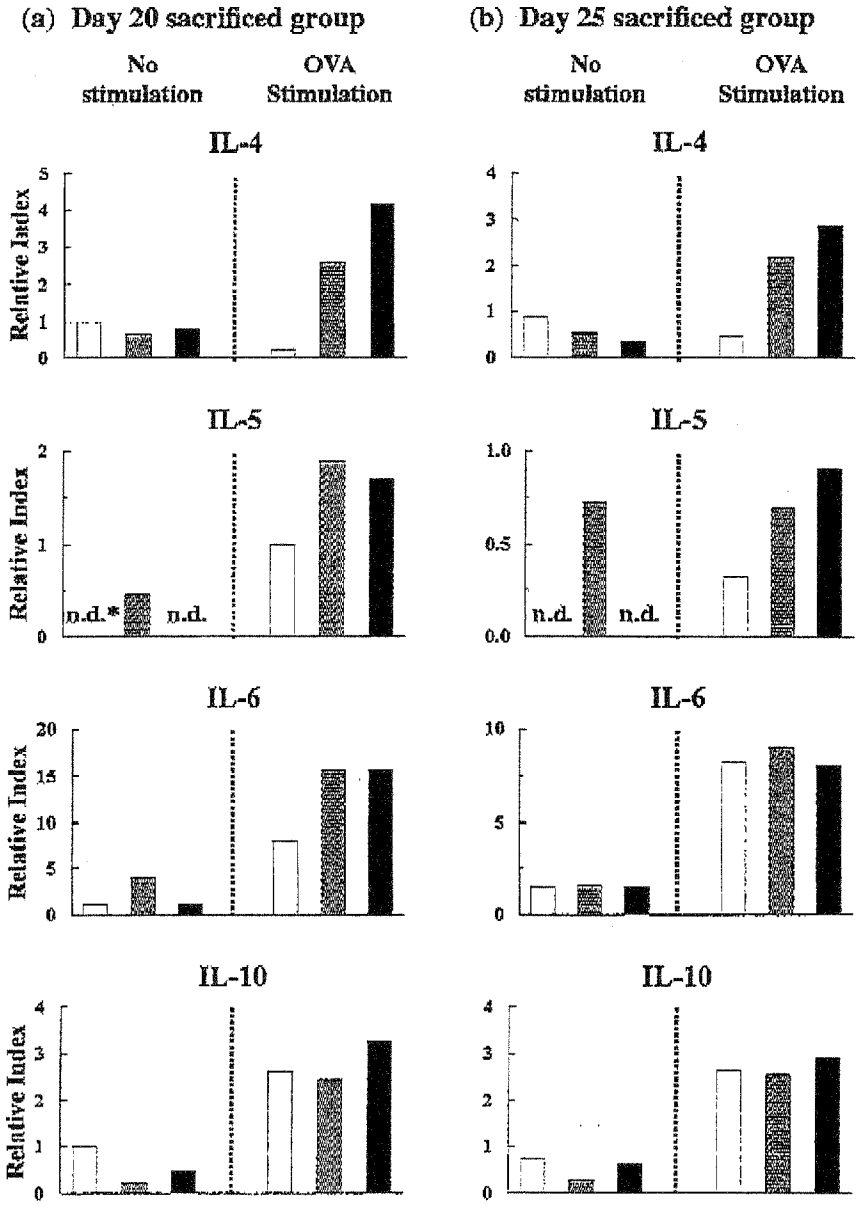

Fig. 5 Gene expressions of Th2 and regulatory type cytokines in preventive study

Control group OVA group P-Dex group , n.d. : Not detected.

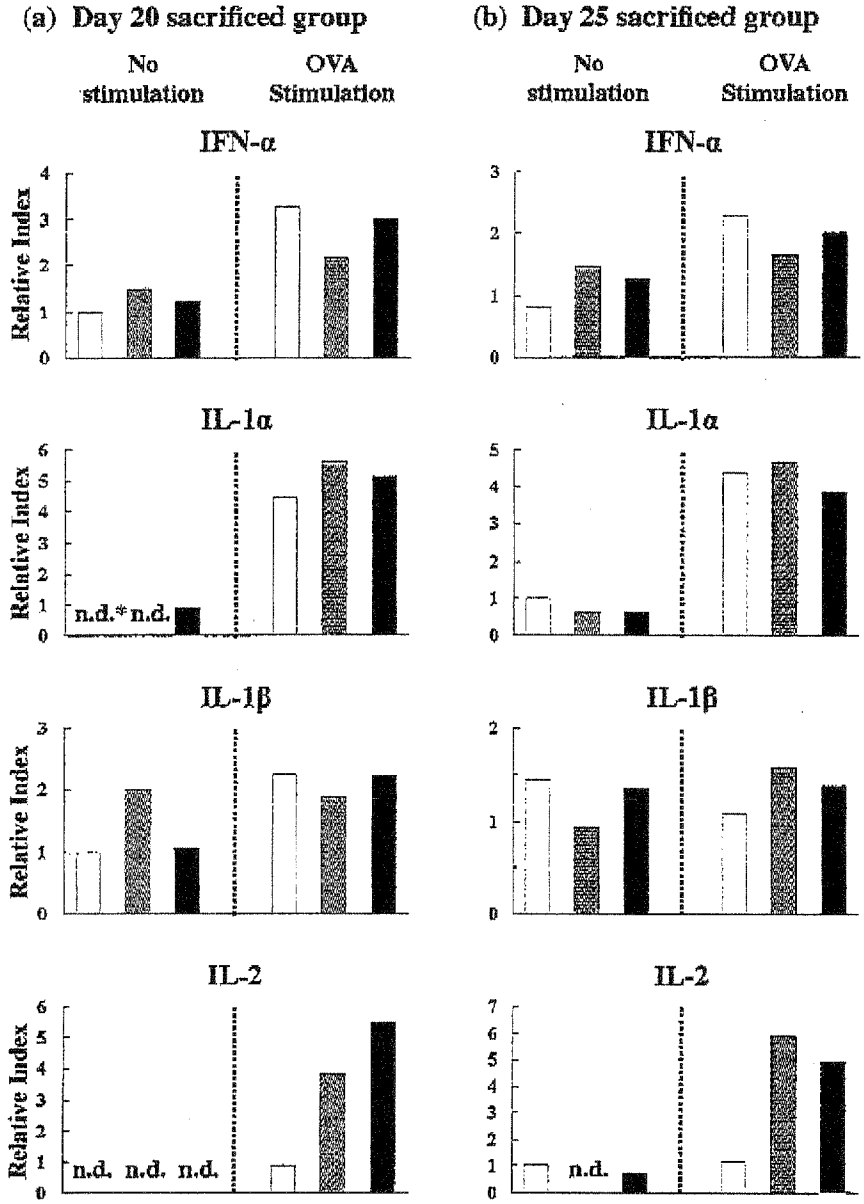

Fig. 6 Gene expressions of the other type cytokines in preventive study

(5) Real-time quantitative PCR analysis of cytokine gene expressions in preventive study

Spleen cells were prepared from the mice in each sensitized group. After 6 hours of *in vitro* stimulation with OVA antigen (100 μg/ml) or without stimulation, the expression of

cytokine mRNAs was analyzed by real-time quantitative PCR. Relative Index for each cytokine was calculated by taking as 1 the intensity in the control group (without stimulation) sacrificed on day 20. The results are shown in Fig. 7.

In vitro OVA stimulation strongly induced the expression of IFN-γ mRNA in all groups. In both groups sacrificed on days 20 and 25, the expression of IFN-γ mRNA was induced in the P-Dex group as compared to the control and OVA groups. When no stimulation was added, the expression level of IL-12p40 in the P-Dex group was higher than that in the control group but lower than that in the OVA group. Meanwhile, when the cells were stimulated with OVA *in vitro*, the mRNA expression of IL-4 was more induced in the P-Dex group compared to the control and OVA groups. *In vitro* OVA stimulation significantly increased the expression level of IL-10 mRNA in the P-Dex group as compared to the other two groups, regardless of the day of sacrifice (day 20 or 25).

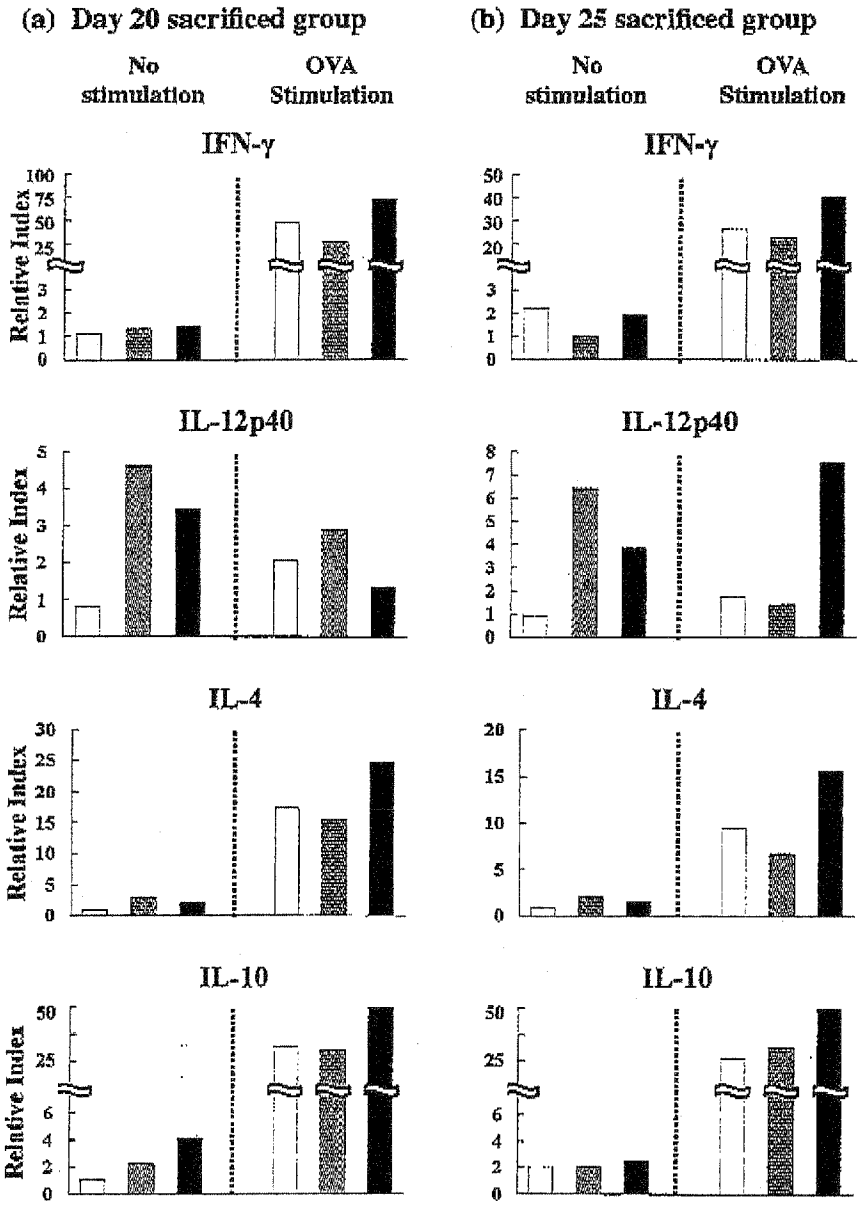

Fig. 7 Real-time quantitative PCR analysis of cytokine gene expressions in preventive study